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Tightening of Gelatin Chemically Cross-linked Networks Assisted by Physical Gelation

Marcelo A. da Silva,[a] Jie Kang,[b] Tam Bui,[c] Lisa Borges da Silva,[d] Jake Burn,[d] Joseph L. Keddie,[d] and Cécile A. Dreiss[b]*

[a] University of Bath, Dept. of Chemistry, Claverton Down, BA2 7YA, Bath, UK

[b] King's College London, Institute of Pharmaceutical Science, 150 Stamford Street, London SE1 9NH, UK

[c] King's College London, Biomolecular Spectroscopy Centre, Pharmaceutical Optical & Chiroptical Spectroscopy Facility, The Wolfson Wing, Hodgkin Building, London SE1 1UL, UK

[d] University of Surrey, Department of Physics, Guildford, Surrey GU2 7XH, UK

Correspondence to: Cécile A. Dreiss (E-mail: *cecile.dreiss@kcl.ac.uk*)

ABSTRACT

Developing the use of polymers from renewable sources to build hydrogels with tailored mechanical properties has become an increasing focus of research. The impact of the thermo-reversible physical networks of gelatin (arising from the formation of triple-helices) on the structure formation of a chemical network, obtained by cross-linking with glutaraldehyde (a non-catalytic cross-linker), was studied using optical rotation, oscillatory rheology and large strain mechanical deformation. We observed a direct correlation between the storage shear modulus of the chemical network grown in the gel state (i.e. simultaneously with the physical network) and the amount of gelatin residues in the triple-helix conformation (χ). Since χ is directly affected by temperature, the value of the storage modulus is also sensitive to changes in the temperature of gel formation. χ values as low as 12% lead to an increase of the shear storage modulus of the cross-linked gel by a factor of 2.7, when compared to a chemical network obtained in the sol state (i.e. in the absence of a physical network). Our results show that the physical network acts as a template, which leads to a greater density of the chemical cross-links and a corresponding higher elastic modulus, beyond what is otherwise achieved in the absence of a physical network.

KEYWORDS: gelatin, hydrogels, linear rheology, optical rotation, large strain deformation

INTRODUCTION

Gelatin is the denaturated form of animal protein collagen obtained by hydrolytic degradation.[1] Collagen is the most common animal protein and its biological origin imparts several useful characteristics to gelatin-based materials, such as cell-adhesion, biocompatibility, and biodegradability.[2,3] Gelatin offers the particular, advantageous feature of a thermally reversible sol-gel transition. The sol-gel transition arises from the single-strand-to-triple-helix transition of gelatin chains as the temperature is decreased below its melting temperature.[4] In the sol state, the single-stranded form of gelatin prevails, and does not display any extensive, classical elements of secondary structure (α-helix, β-sheet or turns).

During the gelation process, the gelatin single-strands self-associate to reform the original collagen triple-helix based on the left-handed, PII conformation of the individual strands.[5] These triple-helices act as junction zones of the three-dimensional network. This network, however, is transient in nature and can easily be removed by environmental changes. Traditionally, chemical cross-linkers are used in the sol state to obtain permanent networks, a process controlled by the diffusion of the cross-linking partners, which leads to relatively heterogeneous networks.[6-8] Recently, however, it has been reported that conducting the chemical gelation simultaneously with the physical gelation produces hydrogels with a higher shear modulus[8-11] and better transparency[12, 13] than similar hydrogels obtained by cross-linking in the sol phase. This synergistic effect - the increase of the chemical network shear modulus in comparison with the equivalent network obtained in the sol phase - has been tentatively attributed to a templating effect of the physical network, which guides the chemical cross-linking in a way that increases the number of elastically active bonds generated and achieves a more homogeneous network.[8-10, 12]

In this work, we examine further the origin of this synergistic effect. More specifically, we explore the effect of temperature, which controls the extent of triple helices formation (the further below the melting temperature, the higher the number density of helices).[4] Our hypothesis is that a more extensive physical network will induce a more efficient cross-linking process carried out contemporaneously. In contrast with previous studies,[8-10, 12] which had used an enzymatic cross-linker (therefore not consumed by the reaction), we study here the cross-linker glutaraldehyde, to establish whether the performance of a classic, non-catalytic chemical cross-linker is also improved when used in the presence of a physical network. The cross-linking process is performed at temperatures both below and above porcine gelatin's melting temperature. In this way, we can control the amount of physical network present and evaluate its effect on the chemical cross-linking process. Optical rotation is used to measure the amount of triple-helices and oscillatory rheology to monitor gelation and assess the linear viscoelastic properties of the final network. In addition, we extend the study by determining the non-linear viscoelasticity at high strains, which is a direct probe of the network structure.

EXPERIMENTAL

Materials

Type A Porcine skin gelatin (G1890) and glutaraldehyde (G6257 - Grade II, 25% aqueous solution) were obtained from Sigma Aldrich and used as received. The porcine skin gelatin has an isoelectric point of pI 7 to 9, a bloom strength of 300, and a gelation temperature of ca. 37 °C.

Methods

Sample preparation: Gelatin samples were prepared by dilution from a 20% w/w gelatin stock solution in water, at natural pH. Gelatin stock solutions were prepared by adding gelatin to water, leaving overnight at 4 °C to swell, then vortex mixing at 40 °C until a clear solution was obtained. The stock solutions were kept for a maximum of five days to prevent contamination.

Gelation protocols: In this study, three types of gelation protocols were used:

Protocol 1: Physical gels (P). Gelatin solutions (10% w/w) were cooled down from 40 °C to temperatures below gelatin melting temperature, namely, to 15, 20 and 25 °C. In this case, only the physical network forms.

Protocol 2: Chemical gels (C). Glutaraldehyde was added to gelatin samples at 40 °C to obtain solutions of 10% w/w of gelatin and 0.25% w/w glutaraldehyde. The final solutions were left to gel at 40 °C, above the melting temperature. In this case, only the chemical network forms.

Protocol 3: Physical-co-chemical gels (PC). Glutaraldehyde was added to gelatin stock solutions at 40 °C to obtain solutions of 10% w/w of gelatin and 0.25% w/w glutaraldehyde. The samples were then cooled immediately to temperatures below the gelatin melting temperature (to 15, 20 and 25 °C) in order to allow the formation of the physical network. In this case, both chemical and physical networks are formed simultaneously.

For the chemical cross-linking, the solutions of gelatin and cross-linker are briefly mixed together before being loaded into a cuvette (for ORD) or onto the rheometer plate. We estimate that this takes < 30 s prior to the measurement being started. For physical gels instead, gelation is started by dropping the temperature *in situ*.

Oscillatory Rheology

Rheological measurements were performed on a strain-controlled ARES Rheometer (TA Instruments, USA) equipped with 25 mm parallel plates geometry. The temperature was controlled by a Peltier unit (±0.1 °C). To prevent solvent evaporation, a thin layer of paraffin oil was applied around the edges of the geometry.

The storage modulus (*G'*) and loss modulus (*G"*) temporal dependence (time sweep measurements) was monitored at a fixed given temperature and fixed frequency of 6.28 rad·s^{-1} and strain of 1% (within the linear viscoelastic region, as determined by an amplitude sweep performed on the physical gel) for 60 minutes. Time sweep measurements were followed by frequency sweep measurements within the range of 0.1-100 rad·s^{-1} at a fixed strain of 1%. For the systems studied here, the gel point is defined as the point where *G'* reaches the arbitrarily defined value of 1 Pa, as was used previously.[8, 9]

Optical Rotation

Optical rotation dispersion (ORD) measurements were conducted on an Applied Photophysics Chirascan™ instrument (Leatherhead, UK), fitted with a calcite polarizer capable of fast scanning optical rotation dispersion in the wavelength region of 850-215 nm. Temperature was controlled by a Quantum Northwest TC125 Peltier unit attached to the cuvette holder. An OMEGA thermocouple probe (TJC-100-CASS-IM025U-35, supplied/calibrated by APL) was used to directly measure the temperature sample inside the sample cuvette. Samples were loaded in optical quartz Suprasil cells. All cuvettes (10 mm and 1 mm path lengths) used in these measurements were obtained from Hellma UK. Optical rotation of air, pure water and sucrose solution (10 mg/mL, standard) were monitored at 589 nm (sodium D-line) at 20 °C to calibrate the instrument prior to gelation measurements as described elsewhere.[8] The instrument was continuously flushed with pure nitrogen vapours throughout the duration of the measurement. Gelation kinetics were monitored at a wavelength of 436 nm, with a bandwidth of 2 nm, and the water baseline corrected. The concentrations of gelatin and glutaraldehyde were kept at 10% w/w and 0.25% w/w, respectively. The optical rotation value for gelatin (10% w/w) in the single-strand state gelatin, α_{coil}, was measured and obtained at 40 °C. The kinetics of gelation were measured by transferring approximately 400 µL of the 10% w/w gelatin solution (incubated at 40 °C) into an empty 1 mm cuvette which was then thermally heated in the instrument sample holder at the appropriate temperature to be measured. Measurements were taken over 18,000 seconds with an accumulation time of 2 s per point. During gelation, single stranded gelatin undergoes a conformation change to give a triple helix. This can be observed from a change in the optical rotation angle α, which can then be used to derive the fraction of triple helices (χ) in the sample as explained elsewhere.[8] Optical rotation measurements were performed three times for each sample type and the resulting average is shown, unless otherwise stated.

Uniaxial Deformation

To complement the rheological measurements of linear elasticity at small shear deformations, the non-linear elastic deformation under uniaxial tension was investigated. Protocols 1, 2 and 3 described above were followed with some modifications, as outlined hereafter, when preparing samples. To prepare physical gel samples via Protocol 1, 10 mL of a 10% w/w gelatin solution at 50 °C was poured into a polystyrene Petri dish (diameter of 5 cm). The solution cooled naturally at room temperature (ca. 20 °C) and was aged for 24 h at that temperature. The physical gel was then cut into rectangular strips (4 cm × 0.3 cm) for stress/strain analysis. To prepare chemical gel samples, 10% w/w solutions at 50 °C were cast into Petri dishes immediately after adding the glutaraldehyde. The gels were held in a closed dish in a water bath at 50 °C (above the gelatin melting temperature, thus preventing the formation of physical cross-links) for 24 h before cutting strips for stress/strain analysis. To prepare physical-co-chemical gels via Protocol 3, 10% w/w solutions at 50 °C were cast into Petri dishes immediately after adding the glutaraldehyde. In analogy to the procedure for the rheology, the gels were held in a closed dish in a water bath at temperatures of either 20 or 25 °C for 24 h. Additionally, some gels were stored in a closed dish within a refrigerator at 5 °C for 24 h.

Strips of chemical and physical-co-chemical gels were measured under tension on a testing apparatus (TA.XT.Plus Texture Analyser, Stable Micro Systems, Godalming, Surrey) with a constant cross-head speed of 0.25 mm·s^{-1} using a 5 kg load cell. The apparatus was fitted with an enclosed thermal cabinet (TC/LN2, Stable Micro Systems) set at a temperature of 45 ± 5 °C. Each end of the strips was held in a steel clamp with a roughened jaw. Samples were strained until the point of failure. Dishes of water within the oven raised the relative humidity within the oven to suppress evaporation of water from the gel samples. Prior to the measurement, the gel strips were equilibrated to the oven temperature by placing them in a closed Petri dish containing saturated water vapour. For

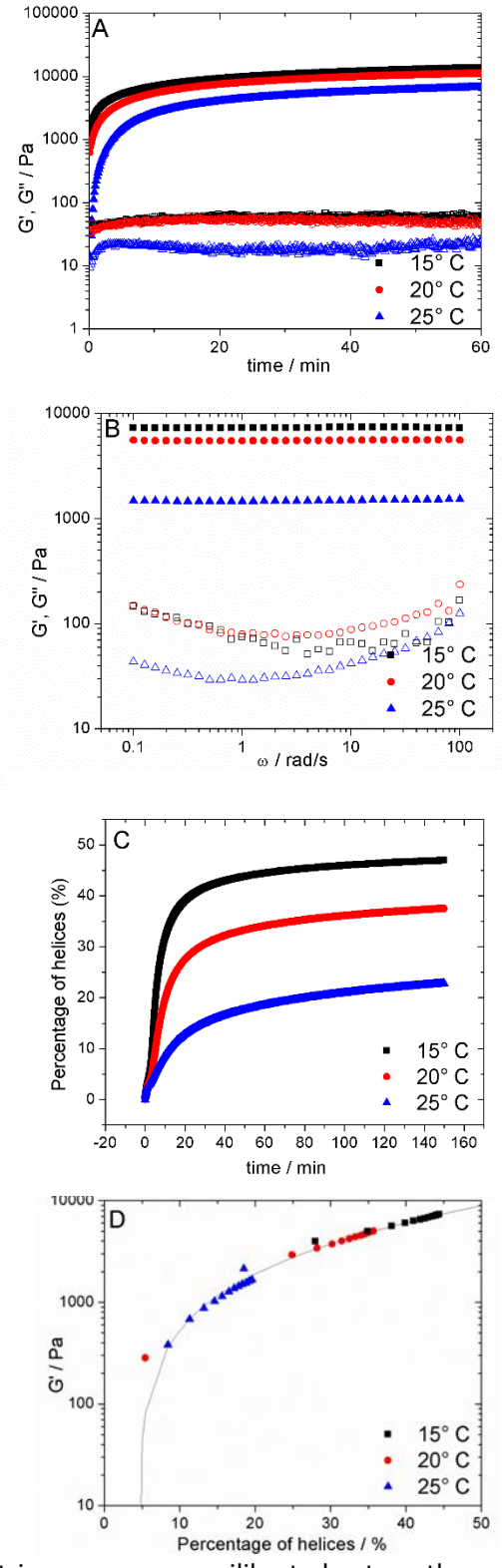

Figure 1. Results from oscillatory rheology and optical rotation for gelatin physical hydrogels (10% w/w) at three different temperatures: (■) 15, (●) 20 and (▲) 25 °C. (A) Time sweeps at a frequency of 6.28 rad·s^{-1}. Filled symbols represent *G'* and open symbols represent *G''* (*G"* curves are below the corresponding *G'* traces). (B) Frequency sweeps. (C) Evolution of the percentage of gelatin residues in triple-helix conformation (χ) over time. (D) Master curve correlating χ with G' (the line is used as a guide to the eye).

comparison, physical gel samples were analysed at room temperature, which is below the gelatin melting temperature.

RESULTS AND DISCUSSION

Physical (P) gels

The results obtained for the pure physical hydrogels is shown first. For the physical gels, only the self-assembled triple-helix network is present. As it is well established, gelatin undergoes a thermo-reversible conformational change as temperature is decreased,[14, 15] whereas the single gelatin strands form triple-helices, which act as the junctions of a physical network that sustains the hydrogel.[4, 16] In Figure 1A, the temporal dependence of *G'* and *G"* (cure curve) is shown at three different temperatures, 15, 20 and 25 °C. As it can be observed, the gelation time is short, less than 0.15 s at 15 and 20 °C and ca. 0.15 s at 25 °C, in all cases below or close to the experimental limit. As reported in the literature, the conformational reorganization reaches a stage where a slow kinetic regime sets-in.[17-19] In the time sweeps, this is observed as a lack of a plateau, whereby *G'* follows an asymptotic behaviour at long times. The dependence of *G'* and *G"* with frequency shows the typical pattern expected for a permanent hydrogel (Figure 1B): *G'* shows no frequency dependence within the window studied and *G'*>>*G"*.[20] This shows that in these physical gels, the long-life of the triple-helix junctions[21] confers a permanent character to the hydrogel. The value of *G'* at long times is strongly dependent on the temperature, but this dependence also follows an asymptotic behaviour. The value of *G'* correlates directly with the fraction of gelatin's residues in the triple-helix conformation (χ) (Figure 1C), which is controlled by temperature, with a dependence that is also asymptotic. This relationship between *G'* and χ allows us to build a master curve (Figure 1D), i.e., a direct correlation between *G'* and χ, which had first been suggested by Djabourov and co-workers[22] and also demonstrated with fish gelatin.[8]

Chemical (C) gels

The gelatin chemical hydrogels were obtained at 40 °C, i.e., above the porcine gelatin melting temperature of 37 °C. Under these conditions, the gelatin single strands are in a dynamic conformational state with a marked tendency to adopt the PII extended conformation, which is a pre-requisite for triple-helix formation.[5, 23] The chemical network arises from the covalent bonds created by the cross-linker, in this case, glutaraldehyde. Glutaraldehyde forms cross-links by reacting one of its aldehyde group with an ε-amine groups from lysine or hydroxylysine residues, forming an intermediate, which then reacts with another ε-amine group to produce a cross-link.[24, 25] For porcine skin gelatin, on average, 0.2% of the residues are hydroxylysine and 2.7% are lysine residues.[26] Assuming an average molecular weight for the amino acid residues of 110 Da, with 10% w/w gelatin and 0.25% w/w glutaraldehyde in the pre-gelation solution, we estimate an approximately equimolar solution of reactant and substrate, therefore about twice the amount of glutaraldehyde required to react with all ε-amine groups available.

The final value of *G'* depends on the cross-link density and on its efficiency. Not every covalent bond generated will create an elastically active chain. Glutaraldehyde molecules bound to a single amine site generate dead-ends and intramolecular bonds will not propagate the network, creating closed loops. These closed

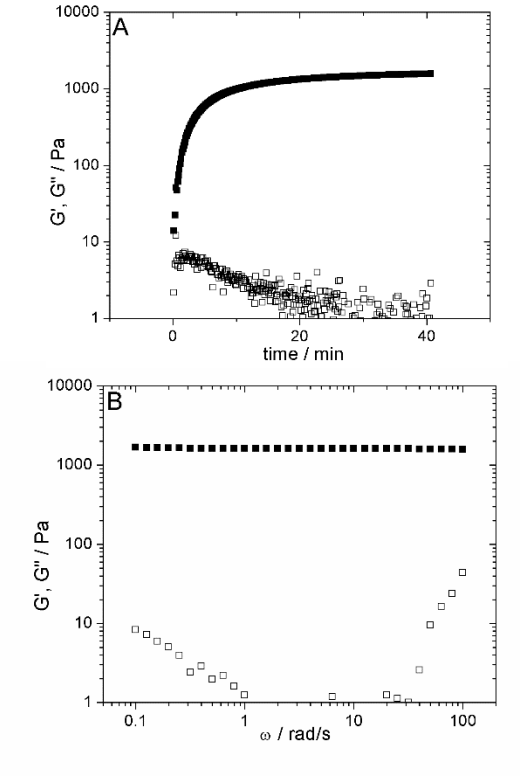

Figure 2. Rheological data for chemical gelatin hydrogels (10% w/w) at (■) 40 °C. (A) Time sweep at a frequency of 6.28 rad·s^{-1}. Filled symbols represent *G'* and open symbols represent *G"*. (B) Frequency sweeps performed on the final gels.

loops and dead, or loose, ends consume glutaraldehyde and binding sites without contributing to the network and therefore to G'. A more detailed discussion can be found in our previously published papers.[8-10, 12] Small-angle neutron scattering data from our previous work with gelatin[10] and gelatin/chitosan[9] hydrogels and data from the literature[27] show a stark difference in the level of homogeneity between physical-co-chemical and chemical networks, in some cases even visible to the naked eye,[12] which suggest a higher contribution of non-elastic chains in the chemical gels.

Figure 2 shows the oscillatory rheology data for the chemical gelatin gels studied in this work, with 10% w/w gelatin and 0.25% w/w glutaraldehyde. The reaction ends when all glutaraldehyde or binding sites have been consumed, or have become inaccessible due to constraints imposed by the network. However, as the cross-linker is consumed and the binding sites are occupied, the reaction will naturally slow down. This slow-down can be observed in Figure 2A, as up to 40 minutes, *G'* values still show a weak, but constant increase. The magnitude of the increase, however, is only about 0.5% between the 39th minute and the 40th minute. In the following, we compare *G'* values at t = 40 min, as being representative of the saturation value. The frequency sweep obtained for this system (Figure 2B) shows the typical behaviour expected for a chemical network: *G'* shows no frequency dependence and *G'*>>*G"*, with very low values of *G"* or close to zero.[20]

Physical-co-Chemical (PC) gels

Physical-co-chemical hydrogels, PC hydrogels, are formed by the simultaneous build-up of both chemical networks, via glutaraldehyde cross-linking, and physical networks, via triple-helix formation. This is achieved by mixing gelatin and glutaraldehyde above the gelatin melting temperature followed by quickly decreasing the temperature to below the gelatin melting temperature (in this work, 15, 20 and 25 °C). The curing curves for the independent physical and chemical reactions were presented in Figures 1A and 2A, showing for both a fairly fast process, with gelation times of the order of 0.15 s or less. Therefore, we can assume that both processes are occurring within the same timeframe, which is in contrast with the enzymatic cross-linker used in our previous work,[8, 10] where the build-up of the chemical network was notably slower than the formation of triple-helices.

In Figure 3A, the time dependence of *G'* and *G"* for the physical-co-chemical hybrid networks is shown. The frequency sweeps are presented in Figure 3B, and show, as already described for both physical and chemical individual networks, the typical signature of hydrogels.

A comparison of the curing curves (Figures 1A, 2A and 3A) reveals both similarities and disparities between the three processes. The gelation is fairly fast, with gelation times below 0.15 s. The final value of *G'* for PC gels depends on the temperature, however it reaches a saturation limit faster than the physical gels. For P gels (Fig. 1A), the value of *G'* increases by ca. 3.2 times by decreasing the temperature from 25 to 20 °C and 1.5 from 20 to 15 °C. For the PC gels (Fig. 3A), *G'* increases by 1.8 and 1.2 times from 25 to 20 °C and from 20 to 15 °C, respectively. This earlier saturation of the PC gel is probably an effect of the chemical network setting in: as the chemical network builds up, covalent bonding of the chains may compete with the formation of the physical network, and therefore, limit how much of the physical network can be formed. In contrast with our previous work with an enzymatic cross-linker, transglutaminase,[8, 10] the reaction kinetics with glutaraldehyde are much faster - comparable to the physical gelation – thus leading to a more rapid stabilisation of the storage modulus.

As can be seen in Figure 3C, this early saturation of *G'* does not match the change in the fraction of residues in helix conformation (χ): χ does still exhibit an increase while *G'* reaches saturation, which shows a decoupling of *G'* and χ, in other words, not every triple helix junction formed (which increases χ) leads to an increase in *G'* (earlier saturation). Therefore, it was not possible to build a master curve between *G'* and χ for the PC hydrogel, as the contribution of the chemical network could not be accounted for. Another striking effect of the chemical network is the reduction of χ at any given temperature. For physical hydrogels at 8000 s, χ values are 47, 37 and 22% at 15, 20 and 25 °C, respectively (Fig. 1C). For PC hydrogels, these are: 32, 21 and 12%, at the same temperatures. These much lower values of helix density demonstrate that the formation of the chemical networks hinders the helical conformation change. Thus, we have observed that in the PC gels a decoupling between χ and *G'* takes place: not all triple-helix junctions contribute to *G'* and a smaller fraction of the gelation residues forms triple-helices.

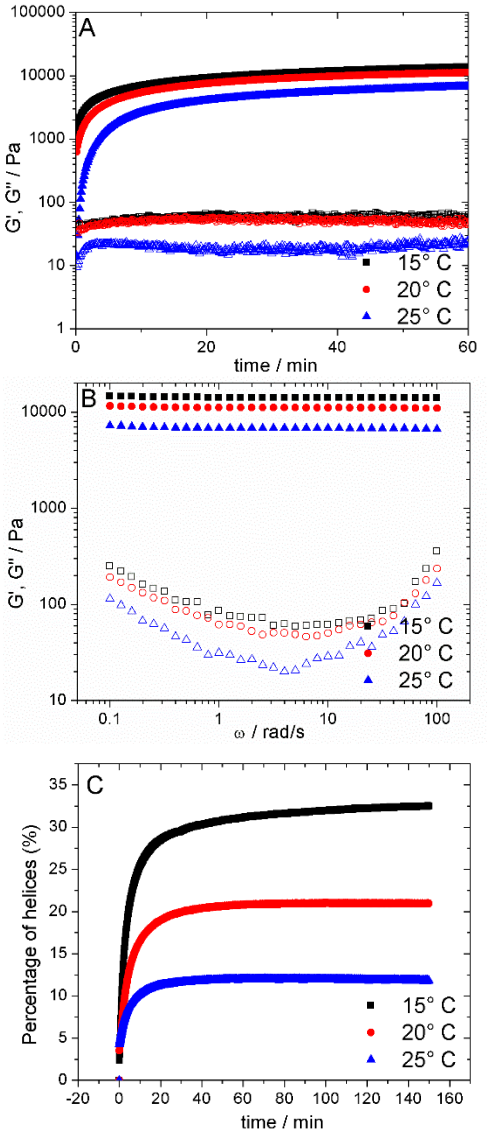

Figure 3. Results from oscillatory rheology and optical rotation for the physical-*co*-chemical gelatin hydrogels at (■) 15, (●) 20 and (▲) 25 °C. (A) Time sweeps showing *G'* and *G''* (*G''* curves are below the corresponding *G'* traces). (B) Frequency sweeps. (C) Percentage of gelatin residues in triple-helix conformation over time.

The presence of both chemical and physical networks in these hybrid gels is also apparent when comparing the magnitude of the modulus.

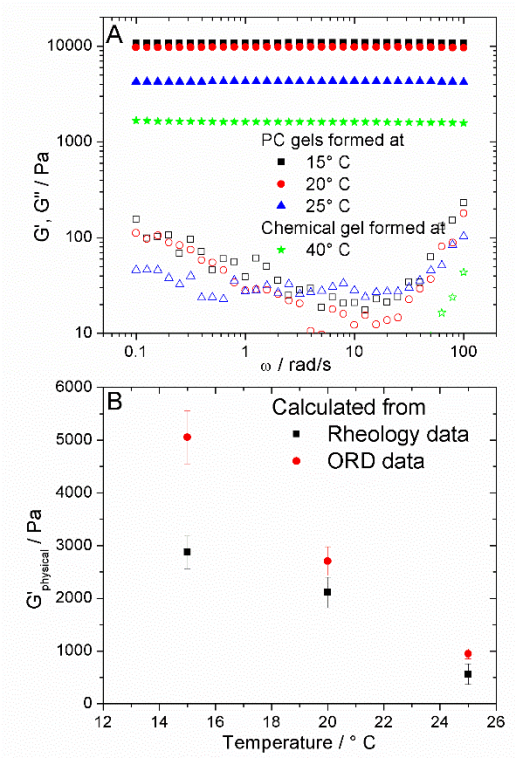

Figure 4. Results for the chemical networks measured at 40 °C, built-up either alone or in the presence of triple-helices at lower temperatures (PC gels). (A) Frequency sweeps of physical-*co*-chemical hydrogels formed at 15 (■), 20 (●) and 25 °C (▲) and chemical hydrogel formed at 40 °C (★). All gels are measured above the gelatin melting temperature, at 40 °C, thus in the absence of physical cross-links formed by the triple-helices. (B) Estimated values of the physical network contribution to the physical-*co*-chemicals obtained from the master curve (●) (Figure 1D) and from the decrease of *G'* upon melting the PC hydrogels (■).

For instance, at 15 °C, *G'* for a physical gel is 6.8 kPa (Fig. 1A), while it is 12 kPa (Fig. 3A) for a PC gel. However, as stated before, it is not trivial to separate the contribution from each network. Therefore, in order to evaluate the impact of the physical network on the formation of the chemical network, we measured the rheological properties of the PC gels at 40 °C, a process schematically represented in the Graphical Abstract. At this temperature, the physical network is completely removed, as demonstrated in earlier work,[8] leaving only the chemical scaffold in place. In figure 4A, frequency sweeps of the PC hydrogels measured at 40 °C, but resulting from the hybrid gelation process at 15, 20 and 25 °C, are shown together with the chemical gel obtained at 40 °C (i.e. in the absence of the physical framework). The synergy between both networks is clearly visible, with the chemical networks obtained in the presence of the triple-helices showing substantially higher values of *G'* than the chemical network obtained in the sol phase (Fig. 5). From Figure 5A, storage modulus values are: 11, 9.8, 4.3 and 1.6 kPa for gels formed at 15, 20, 25 and 40 °C, respectively. Two main conclusions can be drawn from these data. One, the magnitude of the effect depends on the gelation temperature, and thus on the fraction of triple helices present in the physical gel phase: the larger that χ is, then the greater is the increase in the *G'* of the final chemical networks. In the following discussion, we refer to this storage modulus from the chemical networks grown with the PC protocol as $G'_{C@PC}$, to differentiate it from the total *G'*, which includes the contribution from the helices. $G'_{C@40°C}$ refers instead to the modulus from the pure chemical network, i.e. grown at 40 °C, in the absence of triple-helices. While the temperature clearly impacts $G'_{C@PC}$, a saturation, however, is reached when lowering the temperature: at 25 °C, the PC gel's storage modulus is 2.7 times higher when compared to the chemical gel; at 20 °C $G'_{C@PC}/G'_{C@40°C}$ ratio is 6.1, and at 15 °C, it is 6.7 (Fig. 5C). Assuming that this synergistic effect is due to a templating effect of the physical network favouring intermolecular bonding (which contributes to *G'*) at the expense of intramolecular bonding (which does not contributes to *G'*), one can expect that there is an optimal templating architecture which cannot be further improved. Therefore, there exists (for a given composition) an optimum value of χ that leads to a maximum synergy with the chemical network formation, beyond which no further improvement is obtained. Within the conditions

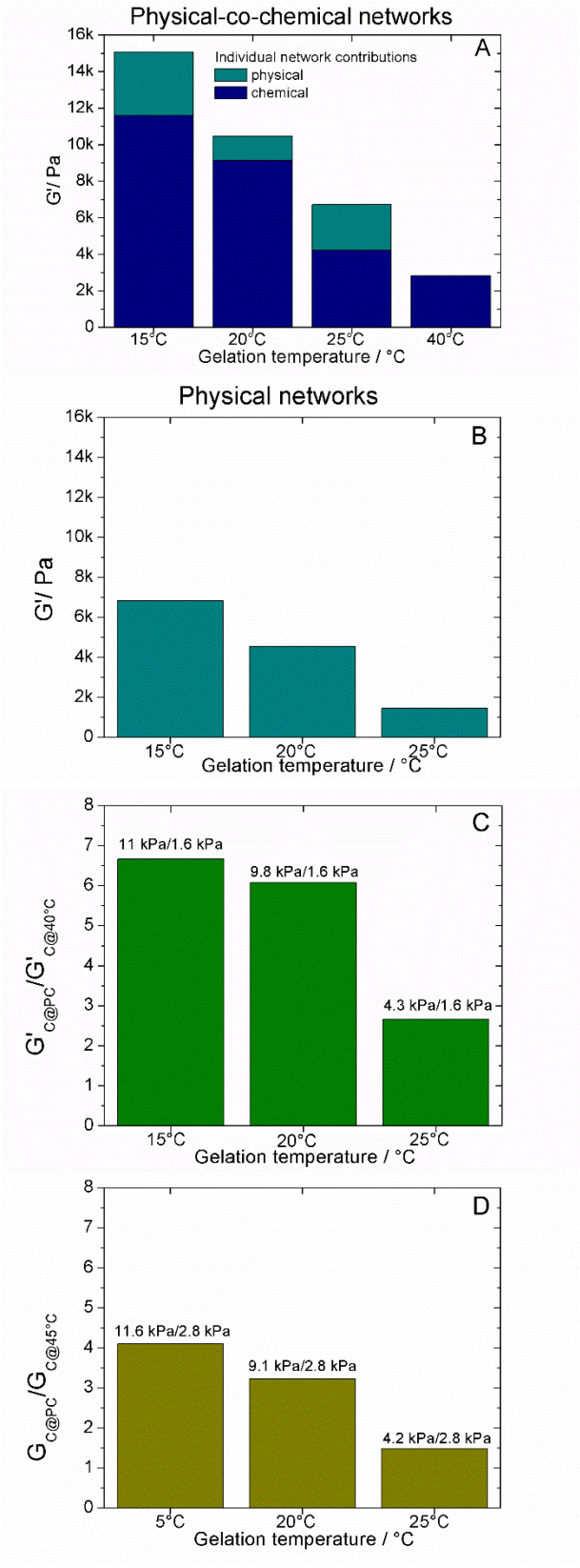

Figure 5. (A) Contribution to the total value of *G'* from the chemical (G'_C) and physical (G'_P) networks in physical-*co*-chemical gels (formed at temperatures of 5, 15 or 25 °C) and *G'* of pure chemical gels (formed at 40°C); the chemical contribution ($G'_{C@PC}$) is measured after melting the helices at 40°C and G'_P is obtained from the difference with the total modulus of the gels, as obtained from frequency sweeps. (B) For comparison, elastic modulus from the pure physical hydrogels. (C) The ratio of $G'_{C@PC}/G'_{C@40°C}$ obtained from oscillatory shear of gels formed at three different temperatures. (D) The ratio of $G_{C@PC}/G_{C@45°C}$ obtained from uniaxial deformation of gels formed at three different temperatures.

studied here, the highest elasticity was obtained for a value of χ around 30% at 15°C. The second important aspect that can be read from these data is that a small amount of triple-helix present is enough to cause a dramatic increase in $G'_{C@PC}$. At the lowest χ value of 12% (obtained at 25° C), $G'_{C@PC}/G'_{C@40°C}$ is 1.5.

In Fig. 4B, the physical contribution to *G'* in the PC gel (obtained from the amount of *G'* lost during melting) is compared with the expected value obtained from optical rotation (Fig. 3C), using the master curve established in Fig. 1D. If there was a direct correlation between χ values measured in PC gels and the physical contribution to the total *G'* in the same hydrogels, both sets of data should superimpose. However, this was not observed; instead, the contribution from the physical network (from rheology) is always lower than the expected contribution from the helices (estimated by the master curve). In addition, this discrepancy increases at lower temperatures. This shows that the respective contributions of helices and covalent bonds in a PC gel are not simply additive, but result from a collaborative process.

This "collaboration" of the physical and chemical networks formation has been observed in similar

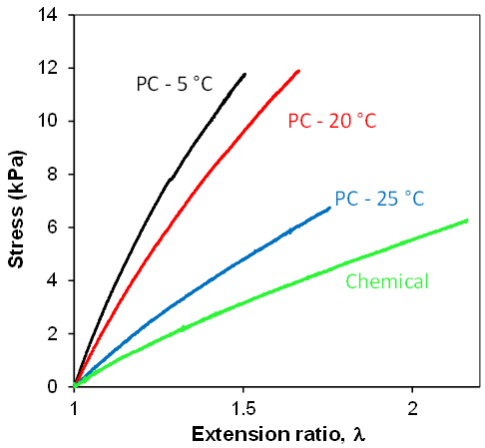

Figure 6. Representative engineering stress data obtained under large-strain extension at 45 °C for physical-*co*-chemical (PC) hydrogels compared to a chemical gel. The PC gels were formed at three different temperatures (as indicated) and measured at 45°C, and the chemical gel was formed at 50 °C.

systems. Kim and co-workers[28] observed that chemical gelatin nanogels, also cross-linked with glutaraldehyde, showed an irreversible volume transition upon melting of the physical network, which was attributed to the irreversible collapse of the chemical network, no longer supported by the physical network. We also observed similar changes in mixed chitosan/gelatin bulk gels, where nano-scale structural re-arrangements were observed upon melting of the physical network.[9] Souguir and co-workers[21] investigated the kinetics of triple-helix formation in the presence of the constraints imposed by covalent cross-links and observed how these constraints affected the physical network kinetics at long times, but not at short times. These constraints imposed by the chemical cross-linking process could explain the lack of a direct correlation between χ and *G'* from the physical network, and why the *G'* vs. χ master-curve becomes a poorer predictor at lower temperatures (Figure 4B), which is also where the chemical networks was observed to be the strongest (post melting of the helices). In a physical network, triple helices are the junctions of the network. Even though it is theoretically possible to have a triple-helix formed by a single looped strand, this is highly unfavourable and generally not observed.[29] Therefore, it can be assumed that all triple-helix junctions are inter-molecular, leading to the highly efficient formation of elastically active chains, and explaining the direct correlation between χ and *G'* in pure physical gels (Fig. 1D). Instead, in hybrid cross-linking processes, the chemical bonds create junctions that, when at high enough concentration, isolate the triple-helices from each other, as observed by Souguir and co-workers.[21] This can create a situation where a triple-helix is formed but does not propagate the network, as it would be isolated from the rest of the network by the chemical cross-links. In this case, not every triple-helix generates an elastically active chain and a decoupling between χ and *G'* would be observed.

Large Deformation of Gelatin Networks

The results obtained at low strains under oscillatory shear can be compared to the results found at large strains under uniaxial stress. These experiments enable comparing the results from the gelatin networks to the expectations from the classical theory of rubber elasticity for entropic, randomly-coiled chains.[7] At high extensions of an elastic network, the chains reach their elastic limit. Then strain hardening is observed; the stress rises more than linearly with strain. Thus, large-strain deformation provides a means to probe a network's structure. In a previous study of cross-linked gelatin,[30] strain hardening was observed at strains greater than approximately 50%.

For uniaxial deformation of a cross-linked network of unentangled chains, the engineering stress, σ (defined as the load per initial cross-sectional area) is related to the extension ratio, λ, as

$$\sigma = G(\lambda - 1/\lambda^2) \quad (1)$$

where *G* is the shear modulus, obtained when invoking the assumption of non-compressibility, a common assumption for hydrogels.[31] The

theory states that G is directly proportional to the density of cross-links (and inversely proportional to the molecular weight of the strands between the cross-linking points).

Figure 6 compares the results for a pure chemical network to PC networks that were formed at three different temperatures: 5, 20 and 25 °C. The networks were strained above the melting point of the gelatin, at a temperature of 45 °C; these experiments therefore probe differences in the chemically cross-linked network structures ($G'_{C@PC}$). The stress/strain data can be well described by Equation 1 for all gelatin network types. The slight curvature observed in the data in Figure 6 is consistent with the predictions of rubber elasticity theory. There is no evidence for strain hardening or stress relaxation from disentangling chains.[7]

The data were fitted to Equation 1 to obtain values of G, which are listed in Table 1. The chemical gel has the lowest G, indicating that it has the lowest cross-link density. The value of G varies inversely with the temperature of the PC gel formation. When the gel is prepared at 5 °C, the ratio of the PC to C moduli, $G_{C@PC}/G_C$, is 4.1 (Figure 5D). As likewise observed from the oscillatory rheology experiments (Figure 5C), this ratio is very sensitive to the temperature where the hybrid gelation process was performed: at 5 °C the ratio is over twice the value found at 25 °C (Figure 5D), thus comparable to the increase observed from the oscillatory experiments,

Table 1. Elastic Modulus and Extension Ratio at Failure for Gels under Uniaxial Deformation.

	(°C)			Energy ($kJ \cdot m^{-3}$)
Physical	20	6.1 ± 0.2	0.37 ± 0.02	1.06 ± 0.11
Chemical	45	2.8 ± 0.2	1.58 ± 0.19	7.56 ± 0.15
PC – 5 °C	45	11.6 ± 0.6	0.63 ± 0.09	5.29 ± 0.12
PC – 20 °C	45	9.1 ± 0.6	0.66 ± 0.09	4.71 ± 0.13
PC – 25°C	45	4.2 ± 0.3	1.24 ± 0.13	6.94 ± 0.14

where $G'_{C@PC}/G'_C$ at 15 °C is twice higher than at 25 °C (Figure 5C). The observed trend in G is broadly consistent with the trend in G' obtained from oscillatory shear rheology at low strains. The experiments clearly show that the density of cross-links increases when the chemical reaction takes place within the template provided by triple helices. Table 1 also presents the strain at failure and the energy expended during deformation (calculated from the area under the stress/strain curves). It is seen that the chemical gel can be extended to greater strains than the PC gel, which can be explained by its lower density of cross-links enabling greater chain extension at relatively low stress levels. Hence, the energy of deformation is greatest for the chemical gel.

It is not possible to compare the deformation of the physical gels to the PC gels at a temperature of 45 °C, as in Figure 5, because of melting. However, data obtained at 20 °C for pure physical gels is included in Table 1. It is noteworthy that the modulus of the PC gel (11.6 kPa, measured at 45 °C) exceeds the sum of the chemical gel's modulus (2.8 kPa) and the physical gel's modulus (6.1 kPa, measured at 20 °C).

CONCLUSIONS

In this work, chemically cross-linked hydrogels of porcine gelatin were obtained using glutaraldehyde, a non-catalytic, non-zero-length, bi-functional chemical cross-linker. We compared the rheological and large-strain deformation properties of conventional chemical hydrogels, gelled from the sol phase by the addition of a cross-linker, to hybrid physical-co-chemical hydrogels, gelled in the gel phase, i.e. in the presence of gelatin's natural physical network. Using shear rheology and uniaxial deformation, we compared the resulting chemical networks, obtained after melting of the physical triple-helices that sustain the physical network. In both types of analysis, hydrogels prepared from a hybrid cross-linking process at the lowest temperatures show a synergistic gain in their shear modulus by a factor of four (large strain) to six (shear rheology), when compared

to conventional chemical hydrogels (prepared in the sol state). We found that this synergistic effect directly correlates with the fraction of gelatin residues in triple-helix conformation, which is controlled by the temperature. Therefore, the gain in shear modulus of the chemical networks is a direct result of the amount of physical network present during the chemical network formation. This supports the hypothesis of a templating effect of the physical network guiding the chemical cross-linking in a way to promote the formation of elastically active bonds, which contributes to the shear modulus, at the expense of elastically inactive bonds, which do not contribute to the shear modulus. We also observed that when both physical and chemical networks are present, their contribution to the total shear modulus is not simply additive, but follows a more complex cooperative relation.

These results show a very simple and effective way of making the chemical cross-linking process more efficient, by exploiting the natural physical gelation of gelatin. The research demonstrates that temperature can be used as a handle to optimise the gelation process. The templated cross-linking strategy is a simple concept that should be applicable to other biopolymers that undergo a natural gelation process.

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REFERENCES AND NOTES

1 A. G. Ward, *British Journal of Applied Physics* **1954**, *5*, 85.
2 S. Van Vlierberghe, P. Dubruel, E. Schacht, *Biomacromolecules* **2011**, *12*, 1387.
3 Z. Fan, Y. Zhang, S. Fang, C. Xu, X. Li, *RSC Advances* **2015**, *5*, 1929.
4 M. Djabourov, *Contemporary Physics* **1988**, *29*, 273.
5 A. F. Drake, G. Siligardi, *Biophys. Chem.* **1988**, *31*, 143.
6 A. J. Kuijpers, G. H. Engbers, J. Krijgsveld, S. A. Zaat, J. Dankert, J. Feijen, *Journal of biomaterials science. Polymer edition* **2000**, *11*, 225.
7 M. Rubinstein, R. H. Colby, "Network and gels", in *Polymer Physics*, Oxford University Press, Oxford, 2003, p. 253.
8 F. Bode, M. A. da Silva, A. F. Drake, S. B. Ross-Murphy, C. A. Dreiss, *Biomacromolecules* **2011**, *12*, 3741.
9 M. A. da Silva, F. Bode, I. Grillo, C. A. Dreiss, *Biomacromolecules* **2015**, *16*, 1401.
10 F. Bode, M. A. da Silva, P. Smith, C. D. Lorenz, S. McCullen, M. M. Stevens, C. A. Dreiss, *Soft Matter* **2013**, *9*, 6986.
11 D. Hellio-Serughetti, M. Djabourov, *Langmuir* **2006**, *22*, 8516.
12 M. A. da Silva, F. Bode, A. F. Drake, S. Goldoni, M. M. Stevens, C. A. Dreiss, *Macromol. Biosci.* **2014**, *14*, 817.
13 H. Souguir, O. Ronsin, V. Larreta-Garde, T. Narita, C. Caroli, T. Baumberger, *Soft Matter* **2012**, *8*, 3363.
14 A. Veis, "*The Macromolecular Chemistry of Gelatin*", Academic Press, London, 1964.
15 J. D. Ferry, *Adv. Protein Chem.* **1948**, *4*, 1.
16 D. Pelc, S. Marion, M. Pozek, M. Basletic, *Soft Matter* **2014**, *10*, 348.
17 W. Harrington, P. H. V. Hippel, *Arch. Biochem. Biophys.* **1961**, *92*, 100.
18 J. L. Gornall, E. M. Terentjev, *Soft Matter* **1988**, *43*, 544.
19 M. Djabourov, J. Leblond, *Journal De Physique* **1988**, *49*, 333.
20 G. M. Kavanagh, S. B. Ross-Murphy, *Prog. Polym. Sci.* **1988**, *23*, 533.
21 H. Souguir, O. Ronsin, C. Caroli, T. Baumberger, *Physical review. E, Statistical, nonlinear, and soft matter physics* **2015**, *91*, 042305.
22 C. Joly-Duhamel, D. Hellio, A. Ajdari, M. Djabourov, *Langmuir* **2002**, *18*, 7158.

23 W. F. Harrington, M. Sela, *Biochim. Biophys. Acta* **1958**, *27*, 24.
24 S. Farris, J. Song, Q. Huang, *J. Agric. Food. Chem.* **2010**, *58*, 998.
25 L. H. H. Olde Damink, P. J. Dijkstra, M. J. A. Van Luyn, P. B. Van Wachem, P. Nieuwenhuis, J. Feijen, *Journal of Material Science: Materials in medicine* **1992**, *6*, 460.
26 J. E. Eastoe, A. A. Leach, "Chemical constitution of gelatin", in *The science and technology of gelatin*, A.G. Ward and A. Courts, Eds., Academic Press, New York, 1977, p. 73.
27 Z. Yang, Y. Hemar, L. Hilliou, E. P. Gilbert, D. J. McGillivray, M. A. K. Williams, S. Chaieb, *Biomacromolecules* **2016**, *17*, 590−600.
28 S. S. Gandhi, H. Yan, C. Kim, *ACS Macro Letters* **2014**, *3*, 1210−1214.
29 L. Guo, R. H. Colby, C. P. Lusignan, T. H. Whitesides, *Macromolecules* **2003**, *36*, 9999.
30 R. D. Groot, A. Bot, W. G. M. Agterof, *J. Chem. Phys.* **1996**, *104*, 9202.
31 S. R. Caliari, J. A. Burdick, *Nat Meth* **2016**, *13*, 405.

GRAPHICAL ABSTRACT

Marcelo A. da Silva, Jie Kang, Tam Bui, Lisa Borges da Silva, Jake Burn, Joseph L. Keddie, and Cécile A. Dreiss

Tightening of Gelatin Chemically Cross-linked Networks Assisted by Physical Gelation

TEXT By performing the chemical cross-linking of gelatin at temperatures where physical gelation also occurs, stronger chemical networks can be obtained. It is hypothesized that the triple-helices, which act as junctions in the physical gels, induce a templating effect for the chemical cross-linking process. By decreasing the temperature, the density of triple-helices increases, subsequently the density of chemical cross-links also increases.

GRAPHICAL ABSTRACT FIGURE